ION TRANSPORT AND SUB-CELLULAR COMPARTMENTATION IN MAIZE ROOT TISSUE AS EXAMINED BY *IN VIVO* ^{133}CS NMR SPECTROSCOPY

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ABSTRACT: ^{133}Cs NMR has been used to examine the intracellular and extracellular pools of Cs^+ containing, and CsCl-perfused, excised maize seedling roots. The ^{133}Cs spectrum is sensitive to ionic environment and the presence of protein. In the absence of shift reagents, resonances corresponding to sub-cellularly compartmented Cs^+, in addition to extracellular Cs^+, were observed. The designated assignments for each intracellular shift were established from chemical shift, spin lattice relaxation times and compartment volume ratios for different root tissue sections. Influx studies of the perfused maize tips showed a rapid uptake of Cs^+ into the cytoplasm followed by a much slower movement into the vacuole. The rate constant for the cytoplasmic efflux was found to match the rate constant corresponding to vacuolar influx. Overall, Cs^+ influx into the tissue was suppressed under hypoxia. Perfusion of Cs^+ -containing tips with K^+ demonstrated that cytoplasmic Cs^+ exchanged directly for K^+ while vacuolar Cs^+ levels remained constant.

INTRODUCTION

With the exception of the pH dependent ^{31}P resonance, no other NMR sensitive nucleus has been used to observe sub-cellular compartmentation in vivo. In combination with paramagnetic metal ions such as Mn^{2+}, broadening of the separate vacuolar and cytoplasmic Pi resonances can give information concerning the amount of Mn^{2+} located in these plant cell compartments (1,2) Although shift reagents such as dysprosium tripolyphosphate can effectively shift the resonances

of Na^+, K^+, or Rb^+ corresponding to the extracellular environment relative to those that are intracellular (3,4), they cannot allow one to decipher the ion partitioning inside the plant cell. Also, ions such as Na^+, K^+, or Rb^+ have a high degree of magnetic resonance invisibility due to strong quadrupolar lattice interactions *in vivo*. Thus it is difficult to account for their intracellular concentrations using this methodology (5,6,7).

Unlike the other alkali metals, ^{133}Cs has very weak quadrupolar lattice interactions and is consequently 100% NMR visible. In addition, its chemical shift is highly sensitive to the concentration of counterions but relatively insensitive to the presence of other cations (8). Davis *et al* (9) have recently demonstrated that one can observe extracellular and intracellular Cs^+ in red blood cells by using ^{133}Cs NMR without the use of shift reagents. In addition, Cs^+ uptake and inhibition of uptake by ouabain was found to be analogous to K^+ (9).

In this study we describe the assignment of the ^{133}Cs NMR spectra of sub-cellularly compartmented Cs^+ in excised maize root tips. In addition, we demonstrate how this methodology can be used to directly assess the movement, exchange and compartmentation of Cs^+ under different conditions.

MATERIALS AND METHODS

Plant Tissue: Maize (*Zea mays* BF-43) root tips (~700, 3-5 mm) were excised from 3-day-old seedlings grown at 27°C in trays lined with paper soaked with 0.1 mM $CaCl_2$. For experiments used to determine compartmentation of CsCl, the 3-day-old seedlings were grown in trays, as above, containing 0.1 mM $CaCl_2$ and 10 mM CsCl. Excised root tips (3-5 mm) and sections excised from the mature root just below the seed (3-5 mm), were harvested for experiments to establish the identity of the two resonances representing the cytoplasmic and vacuolar compartmented Cs^+.

NMR Experiments: All spectra were obtained with a JEOL GX 400 NMR spectrometer operating at 9.4T corresponding to a ^{133}Cs NMR frequency of 52.3 MHz and a ^{31}P frequency of 161.7 MHz. ^{31}P spectra were routinely obtained prior to and following the examination of the ^{133}Cs spectra to verify the energetic state of the perfused tissue. The perfusion system, which included 1000 mL of perfusion medium, was essentially the same as previously reported as were the acquisition parameters used to obtain the 27 min ^{31}P spectra (2). A state of

hypoxia was created by bubbling N_2 as opposed to O_2 through the perfusion medium for approximately 1.5-2 h (2). The aerobic and hypoxic state of the tissue was established from ^{31}P spectra taken during the initial and final periods of the experiments [10]. ^{133}Cs spectra were acquired with either a 90° pulse (27.4 μsec) and a pulse delay of 30 sec or with a 60° pulse (17 μsec) and a pulse delay of 22 sec to obtain quantitative responses for all resonances. Spectral widths were 1000 Hz, with 4k data points and either 10 or 3 Hz digital line broadening. Each of the intracellular resonance areas was quantified with a (Gauss-Newton) non-linear regression curve fitting routine. In Cs^+ influx studies, each spectrum obtained from 32 transients was separated in a stacked experiment by a 1 h waiting period. Following the completion of the experiment, (approximately 12-20 h), the accumulated free induction decays (FID) were stored on disk, normalized to the FID of the first spectrum, Fourier transformed and printed out in sequence. The area of each resonance was evaluated as indicated above. Initial rates of Cs^+ influx were calculated from the slopes of the Cs^+ curve over the first 300 min of perfusion. All spectra were obtained with the spectrometer in an unlocked mode.

The percent visibility of ^{133}Cs by NMR was evaluated on the basis of the area of the ^{133}Cs resonance representing the external 10 mM CsCl. The volume represented by this free circulating solution within the coil volume was 0.35 mL. The area of the external ^{133}Cs resonance was measured without perfusion because the flow of liquid gave an underestimation of area by 10%. The net fresh weight of the excised tips within the coil volume was approximately 1 g. The determination of total Cs^+ within the tips was made with a Perkin-Elmer atomic absorption spectrophotometer and a Cs^+ hollow cathode lamp. The values found for NMR and atomic absorption measurements were 2912 and 3113 μmoles/g fresh weight, respectively.

Spin lattice relaxation (T_1) measurements were performed by the inversion recovery method (180°-T-90°) with and without perfusion to determine the effect of perfusion on the T_1 of the Cs^+ in the circulation medium. Acquisition parameters were as above, with a 90° pulse of 27.4 μsec, and a delay between acquisitions of 50 sec (48 sec pulse delay and 2 sec acquisition). The data were evaluated by a three parameter nonlinear regression analysis.

In vitro ^{133}Cs chemical shift experiments were performed using a 10 mm dual concentric tube assembly (11). The exterior section contained 10 mM CsCl and the

interior tube contained 10 mM CsCl plus the additional solute to be added *i.e.*, KCl, KH_2PO_4, K_2HPO_4, KNO_3, or ovalbumin at pH 3, 4.6, and 7.5. The acquisition parameters used were as described above for the *in vivo* experiments. Differences in chemical shift due to magnetic susceptibility between inside and outside solutions were negligible relative to measured Cs^+ shifts.

RESULTS AND DISCUSSION

^{133}Cs Shift Assignments: As reported by Halliday et al. (7), the ^{133}Cs resonance is highly sensitive to counterion concentrations. These properties could be easily exploited just as the sensitivity to pH is used to define compartmentation in plant cells (12). Figure 1 shows the chemical shift dependance of ^{133}Cs as a function of the concentration of various counterions. Note that while phosphate and chloride ions give downfield shifts to ^{133}Cs, nitrate induces an upfield displacement. The differences in shift slope due to HPO_4^{-2} and $H_2PO_4^-$ is probably a consequence of the ionic strength difference of the two solutions. Chloride is the most abundant intracellular ion in our tissue and we therefore assume that it is responsible for a majority of the observed shift.

We added various amounts of ovalbumin (2.5 - 20%) to a 10 mM solution of CsCl to assess the effect of protein on ^{133}Cs chemical shifts. The observed range for ^{133}Cs shifts at pH 3.0, 6.2, and 8.0 were 1.75 ppm, 1.47 ppm, and 1.76 ppm, respectively. These shifts may be considered important based on the fact that protein concentrations found in the cytoplasm relative to the vacuole are so significantly different (13).

Figure 2 depicts a time course study of Cs^+ uptake in perfused maize root tips. The three ^{133}Cs resonances, I, II, and III are assigned to the cytoplasmic, vacuolar and external Cs^+, respectively. It is assumed that the most downfield shifted resonance I corresponds to Cs^+ in the compartment with the greatest ionic strength and protein content. The next upfield resonance II identifies Cs^+ in a less ionic environment with little protein content (13). Resonance III is produced by the external perfusing solution of 10 mM CsCl. Figure 3 shows the application of the curve fitting routine (see materials and methods) used to resolve the three resonances in a typical spectrum obtained after approximately 20 h of perfusion. Further evidence for the above assignments comes from nuclear relaxation values (See Table 1), which show that the spin lattice relaxation time, T_1, for peak I is

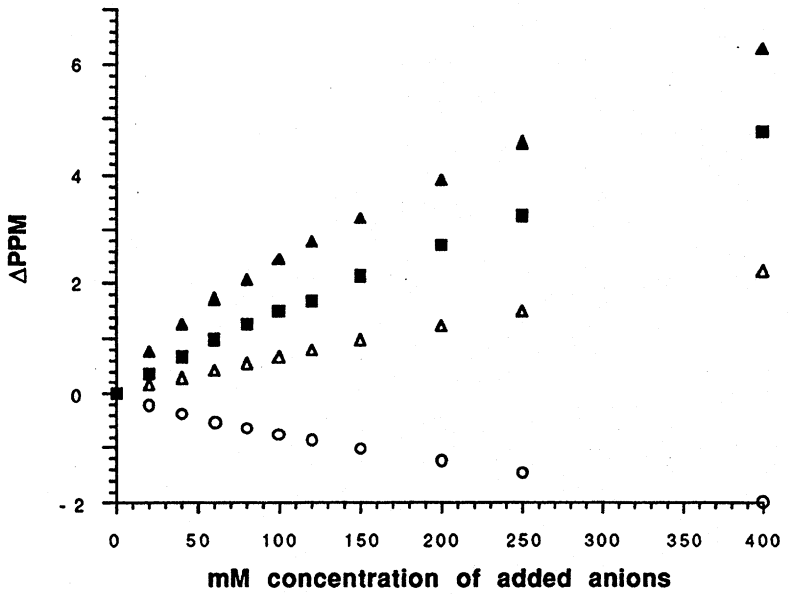

FIGURE 1. ^{133}Cs chemical shifts as a function of anion concentration. ▲ HPO_4^{-2} solution pH 9.1; Δ $H_2PO_4^-$ solution pH 4.4; ■ Cl^- solution; o NO_3^-. *In Vitro* titration experiments were carried out using a 10mm dual concentric tube assembly. The exterior section contained 10mM CsCl and the interior tube, of equal volume, contained 10mM CsCl plus the added ion. Differences in chemical shift due to magnetic susceptibility between inside and outside solutions were negligible relative to the measured Cs shifts.

significantly shorter than for peak II. In addition the T_1 for peak II is essentially the same as that found for extracellular Cs^+ (III). Thus, the vacuolar Cs^+ has a chemical environment much like the extracellular environment, whereas the cytoplasmic Cs which interacts with protein and metabolites in a more restricted state has a significantly shorter T_1.

Differences in the cytoplasm/vacuole volume ratio can also help to establish the identity of each of these resonances. Since it is known that the ratio of the cytoplasm/vacuole volume in corn root tips is 40/60 [4] and on the order of 5/95 (5) in mature root tissue, one can take advantage of the change in the ^{133}Cs

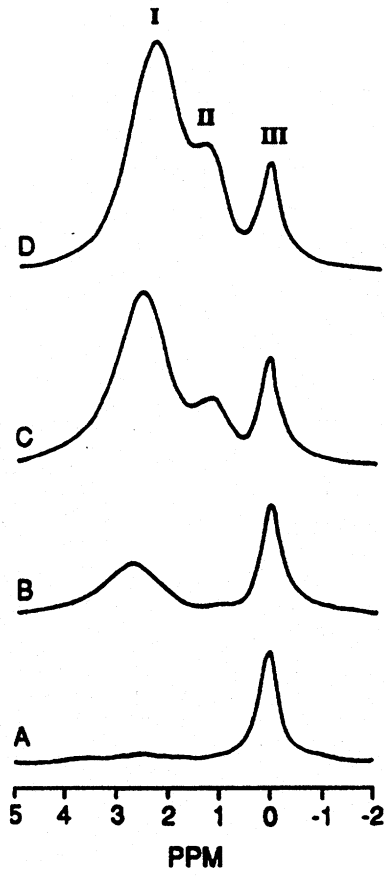

FIGURE 2. 52.3 MHz ^{133}Cs NMR spectra of approximately 600-900 excised (3-5mm) maize (*Zea mays* BF-43) root tips from 3 day old seedlings perfused with 50mM glucose, 10mM CsCl, 0.1mM $CaSO_4$ buffered with MES and Bis-Tris Propane to pH 6.0. Acquisition parameters; 4K data points, spectral width = 1000 Hz, 90° pulse = 7.4 μsec, pulse delay 30 sec, 32 transients per spectrum, exponential line broadening 10 Hz. (A) 19 min after adding the 10mM CsCl to the perfusion medium (B) 1h, 30min after perfusion with the medium containing 10mM CsCl; (C) 7h, 45min of perfusion and (D) 15h, 15min of perfusion.

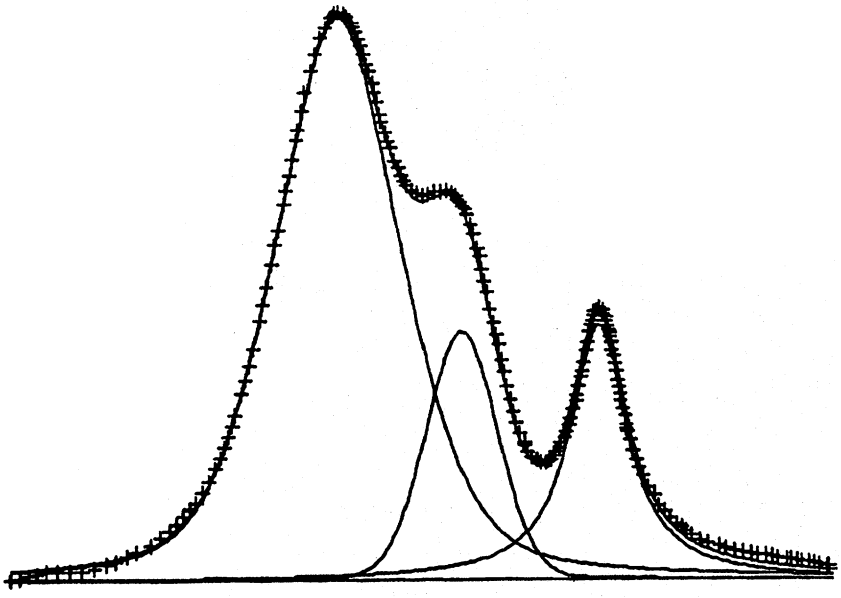

FIGURE 3. Non-linear regression analysis curve fit of a digitized 52.3 MHz ^{133}Cs spectrum of maize root tips following approximately 20h of perfusion with 10mM CsCl at 21° as in Figure 2.

TABLE 1. Characteristic NMR Properties of Intracellular ^{133}Cs Resonances I and II of Excised Maize Root Tips Perfused With 10 mM CsCl, and the Extracellular ^{133}Cs Resonance, III.

	In Vivo		
	I cytoplasm	II vacuole	III external
Relaxation Time T_1 in sec	1.91 ± 0.04	6.09 ± 0.09	6.12 ± 0.23
Chemical Shifts in ppm	2.35	1.08	0
Linewidth in Hz†	42	27	11

† Lines contain 10Hz Computer Line Broadening

resonance ratio in these morphologically different tissues to make shift assignments. An earlier ^{31}P NMR study of Roberts et al. (12) demonstrated that mature maize root tissue contained a greater quantity of vacuolar Pi, cytoplasmic Pi, whereas immature maize root tips showed an almost equal distribution of Pi between both compartments. The inserts in Figure 4 show the ratio of the ^{31}P resonances of the cytoplasmic Pi at 2.6 ppm and vacuolar Pi at ~0.89 ppm in (A) root tips and (B) mature root regions. A similar ratio change is found in the Cs^+ resonances I and II corresponding to Cs^+ in the cytoplasmic and vacuolar compartments of tips (A) and stem sections (B) grown in 10 mM CsCl, respectively (Figure 4).

Ion Uptake and Exchange Studies: A direct assessment of Cs^+ uptake can be made from time course studies as shown in Figure 2. Figure 5 shows a typical Cs^+ uptake curve based on the change in ^{133}Cs resonance areas I and II with time. The intensity of the ^{133}Cs resonance in the cytoplasm first showed a rapid increase and then slowly decreased to a steady level. On the other hand, the increase of Cs^+ in the vacuole appeared to follow a sigmoidal pattern. Three interesting points are noted. First, both signals appeared to reach stable levels at about the same time, ~23 h after perfusion. Secondly, the beginning of the vacuolar Cs^+ increase coincided with the decrease of cytoplasmic Cs^+. And thirdly, the decrease of cytoplasmic Cs^+ from its peak at ~10 h to a steady level is about the same as the total increase of vacuolar Cs^+, suggesting a sequential uptake event. While the exact molecular origin of the first two observations remains unknown, a qualitative mechanism may be offered. With the external Cs^+ concentration at 10 mM, we estimated that cytoplasmic Cs^+ reached about 30 mM before decreasing to a stable level of 20 mM. Assuming that the uptake of Cs^+ across both plasma and vacuolar membranes requires either a simple carrier or channel, the sigmoidal kinetics suggests that the K_m of the tonoplast carrier/channel is much higher than that associated with the plasma membrane. The data also suggest that the tonoplast carrier/channel exhibits an induced positive cooperativity which allows the vacuolar uptake rate to exceed that of cytoplasmic uptake. Since both cytoplasmic and vacuolar uptake of Cs^+ reach stable levels, the following rate (R) relationships should hold:

$$\mathbf{RPi + RVe = RPe + RVi} \quad [1]$$

$$\mathbf{RVi = RVe} \quad [2]$$

$$\mathbf{RPi = RPe} \quad [3]$$

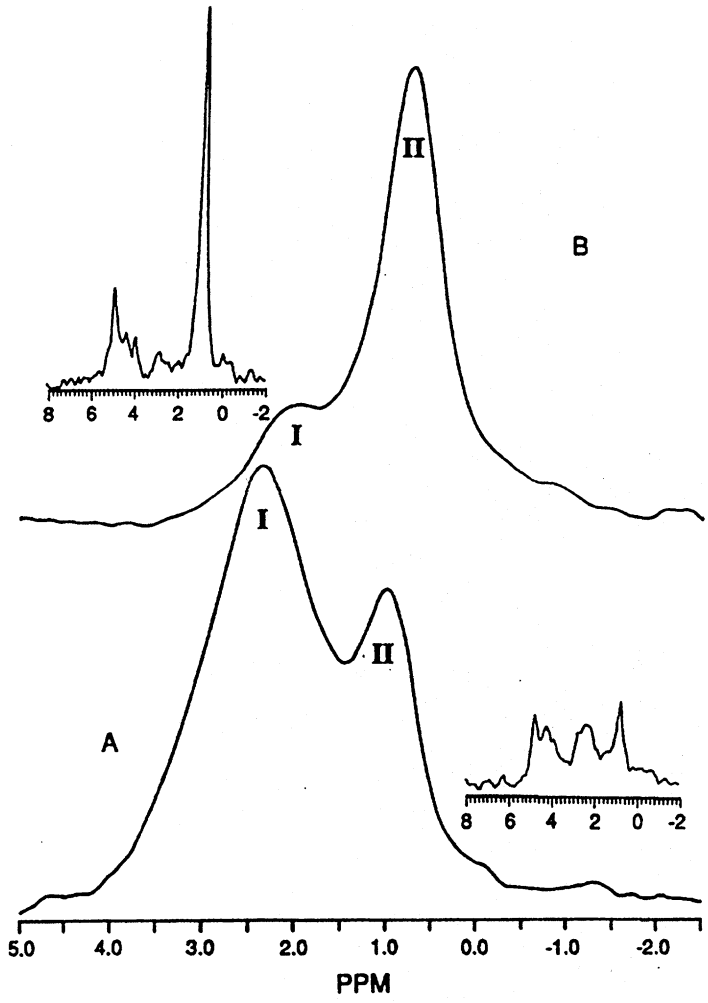

FIGURE 4. 52.3 MHz ^{133}Cs NMR spectrum of approximately 700, 3-day-old excised maize root seedling tips (3-5 mm) grown in 0.1mM $CaSO_4$, and 10mM CsCl solution over wet filter paper (A) and root top sections below the kernel (3-5 mm) (B). Both spectra were obtained with a 60° pulse, 22 sec delay, a spectral width of 1000 Hz, 4k data points and 10 Hz digital line broadening. The ^{31}P NMR spectra (inserts) of the corresponding root sections were obtained as described previously [2]. The resonance at approx. 2.6 ppm represents P_i in the cytoplasmic compartment and the resonance at approx. 0.9 ppm represents vacuolar P_i. All of the tissue was continually perfused during data acquisition [2].

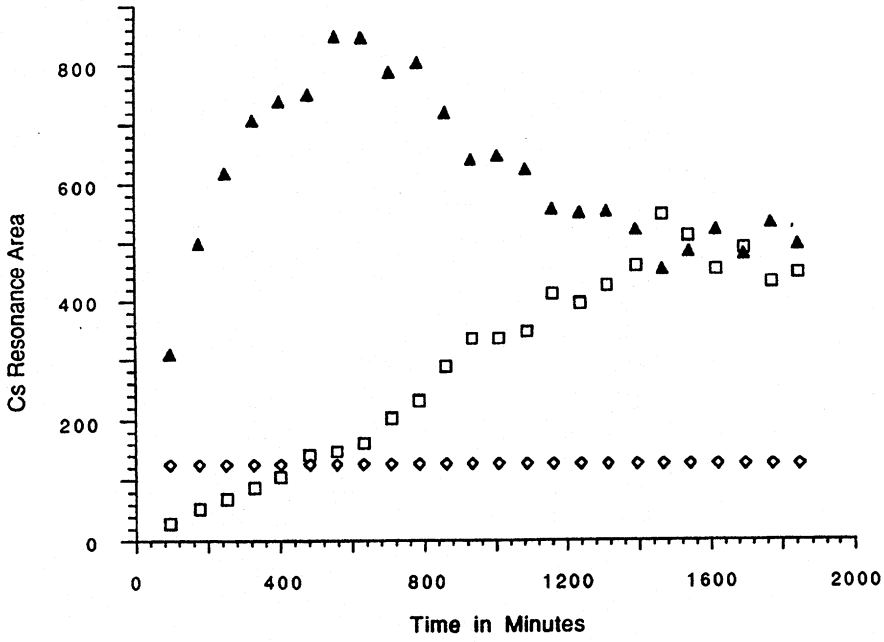

FIGURE 5. Plot of the influx of partitioned intracellular Cs^+ in excised maize root tips as shown in FIGURE 2, as a function of perfusion time. Initial Cs^+ influx rates (cytoplasm: 74.5 nmoles/g fresh wt./min, vacuole: 8.0 nmoles/g fresh wt./ min) were calculated from the first 300 min of perfusion. Curve Δ represents the cytoplasmic compartmented Cs^+ resonance area, the vacuolar compartmented Cs^+ resonance area and ◊ represents the resonance area of the external Cs^+.

in which **P, V, e** and **i** refer to plasma membrane, vacuolar membrane, efflux, and influx, respectively. Equations [1] and [2] delineate the required rate relationships for maintaining cytoplasmic and vacuolar Cs^+ at the steady state. A substitution of [2] into [1] indicates that, at the steady state level, the efflux and influx across the plasma membrane are equal as well [3]. It is interesting to note that unlike the 23 h CsCl perfused excised maize root tips (Figure 5), the 3 day old excised tips derived from seedlings grown on 10 mM CsCl do not show an equal distribution of Cs^+ between the cytoplasm and vacuole but rather a ratio of 1.3 (Figure 4). This is probably a consequence of the fact that not all the Cs^+ that

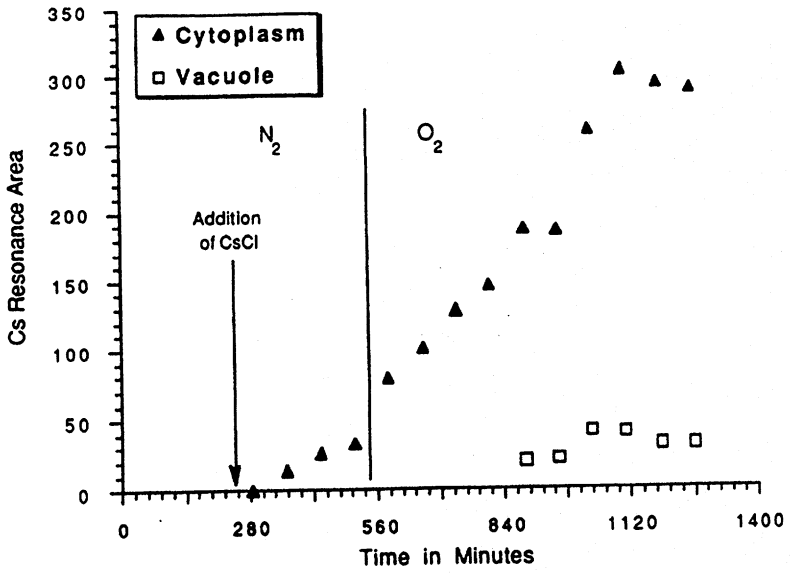

FIGURE 6. Plot of the influx of compartmented intracellular Cs^+ in excised maize root tips during hypoxia and resumption of oxygen perfusion. Top curve represents cytoplasmic compartmented Cs^+ and bottom curve represents vacuolar compartmented Cs^+. Perfusion medium contained 10mM CsCl as in FIGURE 2.

enters the cytoplasm of the intact seedling moves into the vacuole, *i.e.*, some portion can be exported to the leaves via the xylem. Thus the distribution of Cs^+ in the CsCl grown seedlings represents an equilibrium which is physiologically more relevant.

Under hypoxic conditions i.e., nitrogen perfusion for 2 h, Cs^+-uptake was found to be highly suppressed relative to influx under aerobic conditions. (compare Figures 5 and 6). With the resumption of aerobic conditions, the rate of Cs^+ uptake into the cytoplasm increased dramatically, however the vacuolar uptake remained suppressed for 4 h before it could be observed (Figure 6). From these data it appears that the recovery of the energetics responsible for facilitated ion transport in the plasmalemma is more rapid and complete than in the tonoplast. Consequently, the two membranes have different levels of sensitivity to the

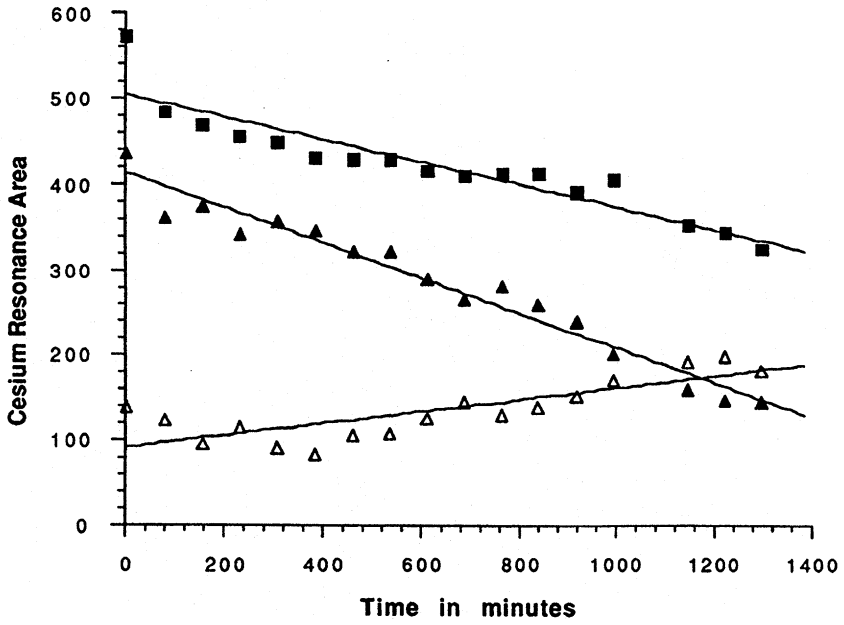

FIGURE 7. Change in ^{133}Cs resonance area in the spectra of CsCl grown maize root tips during perfusion with 10mM KCl at 21° Curve Δ corresponds to vacuolar Cs^+; ▲ corresponds to cytoplasmic Cs^+ and ■ corresponds to the total of cytoplasmic and vacuolar Cs+.

hypoxic conditions. Thus there would appear to be more irreversible damage to the transport mechanisms in the tonoplast relative to the plasmalemma following the resumption of aerobic conditions.

It is presumed that the initial uptake of Cs^+ in maize root tips is associated with an exchange and efflux of K^+ (16). We perfused CsCl grown maize root tips with 10 mM KCl over a period of 21 h in order to establish how these ions exchange. Figure 7 shows that the total amount of intracellular Cs^+ decreased. This decrease is due to exchange with K^+ since control experiments showed no change in the intracellular levels of Cs^+ during this period of time (data not shown). In addition, almost the entire effluxed Cs^+ can be attributed to that which was originally present in the cytoplasm. Vacuolar Cs^+ does not readily exchange

with K^+. A similar phenomenon has been reported for the compartmentation of Na^+ in the vacuole of barley root cells (17) and potentially toxic Mn^{2+} in maize (1,2,10). It would appear that vacuolar trapping of an excess of these "alien" ions may play an important role in maintaining good ionic balance within the cytoplasm.

CONCLUSIONS

Intracellular ^{133}Cs NMR resonances have been assigned to cytoplasmic and vacuolar compartments in maize root tissue. The differences in chemical shift between these resonances have been attributed to the greater ionic strength and higher protein content found in the cytoplasm. Both relaxation measurements and NMR studies of morphologically different sections of the root tissue have verified these assignments. Examination of Cs^+ influx demonstrated that the K_m of the tonoplast carrier is much higher than that associated with the plasma membrane. However, after the establishment of a critical Cs^+ concentration in the cytoplasm, induced positive cooperativity of the tonoplast is observed. Under hypoxia, Cs^+ influx is severely suppressed in both cytoplasm and vacuole. With the restoration of oxygen, the tonoplast, relative to the plasmalemma, is slow to recover, an indication of more severe destruction of the ion transport apparatus in this membrane. Cesium (Cs^+) was preferentially trapped in the vacuole of maize root tips even after an extended period of perfusion with 10 mM KCl. We speculate that competitive protonation of the ion carrier on the vacuolar side of the tonoplast could prevent the Cs^+ from moving back into the cytoplasm (10,18).

We anticipate that ^{133}Cs NMR will be exploited as is ^{31}P NMR to probe the intracellular properties of plant cells. Especially since Cs^+ does mimic K^+ (9). ^{133}Cs NMR may be useful for studying various K^+ modulated processes in cells.

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